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# Notch Signaling Activation in Human Embryonic Stem Cells Is Required for Embryonic, but Not Trophoblastic, Lineage Commitment

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## SUMMARY

**The Notch signaling pathway plays important roles in cell-fate determination during embryonic development and adult life. In this study, we focus on the role of Notch signaling in governing cell-fate choices in human embryonic stem cells (hESCs). Using genetic and pharmacological approaches, we achieved both blockade and conditional activation of Notch signaling in several hESC lines. We report here that activation of Notch signaling is required for undifferentiated hESCs to form the progeny of all three embryonic germ layers, but not trophoblast cells. In addition, transient Notch signaling pathway activation enhanced generation of hematopoietic cells from committed hESCs. These new insights into the roles of Notch in hESC-fate determination may help to efficiently direct hESC differentiation into therapeutically relevant cell types.**

## INTRODUCTION

Human ESCs can divide indefinitely in culture while retaining their pluripotency to form all the cell types derived from ectoderm, endoderm, mesoderm, and trophectoderm (Reubinoff et al., 2000; Thomson et al., 1998; Xu et al., 2002). A major challenge for the future clinical use of hESCs is to efficiently direct ESC differentiation toward specific cell lineages. In addition, it is essential after a directed differentiation procedure to separate committed progenitor cells from residual pluripotent undifferentiated hESCs that may form tumors (teratomas) after transplantation.

The extrinsic and intrinsic mechanisms governing cell-fate choices of hESCs remain obscure. Our current knowledge of hESC-fate determination is largely extrapolated from the studies of mouse embryogenesis and murine ESCs (mESCs). Although human and mouse ESCs share similar fundamental properties such as pluripotency and unique transcriptional networking, they differ significantly in several ways (Ginis et al., 2004; Xu et al., 2002). For example, LIF/STAT3 signaling that is critical for mESC self-renewal is instead dispensable for propagation of undifferentiated hESCs (Daheron et al., 2004; Humphrey et al., 2004). BMP4 together with LIF supports expansion of undifferentiated mESCs (Qi et al., 2004; Ying et al., 2003), while BMP4 induces trophoblastic differentiation of hESCs (Xu et al., 2002). The roles of other signaling pathways in hESCs remain to be fully determined.

The evolutionally conserved Notch signaling pathway plays diverse roles in cell-fate specification in embryogenesis and adult tissues (Artavanis-Tsakonas et al., 1999). In mammalian cells, there are four Notch receptors (Notch1–Notch4) and numerous transmembrane ligands such as Delta-like (i.e., DLL1 and DLL4) and Jagged (i.e., JAG1 and JAG2). Although Notch signaling in organogenesis and murine tissue-specific adult stem cells has been well studied, its precise roles for ESCs are less understood. The Notch signaling network appears active in undifferentiated mESCs (Lowell et al., 2006). After withdrawal of self-renewal factors such as LIF and BMP4 for mESCs in culture, active Notch signaling directed mESC differentiation toward a neuroectodermal fate (Lowell et al., 2006). Multiple *Notch* receptor and ligand mRNAs are detected in multiple undifferentiated hESCs, such as H1 (Brandenberger et al., 2004; Xiao et al., 2006), H7 (Walsh and Andrews, 2003) (Enver et al., 2005), BGN1 (Noggle et al., 2006), and line 181 (Lowell et al., 2006). However, the exact role of Notch signaling in hESCs remains elusive and controversial.

Ligand binding to a Notch receptor results in its cleavage by a membrane-associated protease complex (γ-secretase) containing presenilin (Ehebauer et al., 2006; Ilagan and Kopan,

2007). The released intracellular domain of the Notch receptor (ICN) is then translocated to the nucleus, where it complexes with the DNA-binding protein CBF1 (CSL, RBPJκ, or RBPSUH), the transactivator Mastermind-like (MAML), and other modulators. The complex then binds to the cognate DNA sequence of CBF1 and regulates the transcription of multiple effector genes, including members of *HES*/*HEY* family such as *HES1*. Notch proteolytic cleavage and signaling can be inhibited by γ-secretase inhibitors (GSIs), although they are not specific in blocking Notch activation. Use of a dominant-negative form of MAML1 (DNMAML) provides a second means to block Notch/CBF1-mediated signaling. A DNMAML-GFP fusion protein has been shown to efficiently inhibit CBF1-mediated transcriptional activation by all four Notch receptors in vitro (Weng et al., 2003) and in vivo (Maillard et al., 2004; Tu et al., 2005) and is also used in this study.

Using genetic approaches and a newer GSI with reduced toxicity, we report here that we have achieved both blockade and conditional activation of Notch signaling in two hESCs. We confirmed that the Notch/CBF1 pathway is not required or activated in undifferentiated hESCs. However, Notch signaling activation is required for hESCs to generate derivatives of all three embryonic germ layers, but not the trophoblastic lineage. Based on these novel observations, we propose a new model for the role of Notch signaling in governing hESC-fate choices.

## RESULTS

### Notch Signaling Is Elevated in Differentiated hESCs and Inhibition of Notch Signaling Enhances the Growth of Undifferentiated hESCs as a Population

Consistent with previously published data, we observed that many Notch pathway genes are expressed in hESCs (Table S1 and Figure S1 available online). To directly measure endogenous Notch/CBF1-mediated activity in hESCs, we used a luciferase (Luc) reporter system in which Luc transcription is controlled by the canonical CBF1 responsive element (WT-CBFRE). A related reporter with mutated CBFRE (mutCBFRE) was used as a negative control to determine the basal level of transcription in the same cell types studied. The CBF1 activity in differentiated cells (obtained after teratoma formation) was ~70-fold higher than in undifferentiated cells (Figure 1A). CBF1-mediated activity in undifferentiated and differentiated hESCs was next measured in the presence of GSI-18 that is less toxic than the widely used DAPT (Figure S3). GSI-18 substantially reduced the CBF1-mediated activity of differentiated hESCs, while it had little effect on mutCBFRE reporter activity.

Moreover, we analyzed the endogenous expression of major Notch effector genes including four members of the *HES*/*HEY* family (Figure 1B and Figure S1). As compared to differentiated cells in teratomas (100%), the expression level of all 4 target genes was lower in undifferentiated hESCs (Figure 1B). The expression of the *DNMAML* inhibitory transgene further reduced the expression of *HEY1* and *HEY2*, similar to the findings with the CBF1 reporter assay. Therefore, the Notch signaling pathway is inactive or negligibly low in undifferentiated hESCs.

To further evaluate the functional status of Notch signaling pathway in undifferentiated hESCs, we tested if the exogenous full-length *Notch1* ($N1^{FL}$) cDNA expression could turn on the CBF1 reporter. There was no evidence of Notch cleavage or activation (CBF1 reporter activity) in hESCs after the transfection of the $N1^{FL}$ cDNA (Figure 1C). However, in the presence of exogenous Notch ligand DLL1, 6-fold increase of CBF1 activity was observed only in hESCs transfected with the $N1^{FL}$ cDNA. Interestingly, functional JAG1 treatment (Figure S2C) did not lead to active Notch1 cleavage. Our data confirm that Notch signaling pathway is inactive in undifferentiated hESCs, but can be activated if both exogenous Notch1 receptor and ligand (DLL1) are provided.

Next we examined the kinetics of Notch signaling activation in differentiating hESCs (Figures 1D–1F). A standard method to differentiate ESCs is to form embryoid bodies (EBs) in the presence of serum. First, we transfected a CBFRE-GFP reporter plasmid (Duncan et al., 2005; Mizutani et al., 2007) into undifferentiated hESCs, which were subsequently induced to differentiate by EB formation. Positive GFP expression started to appear one day after EB formation and further intensified during the culture (Figure 1D). In contrast, no GFP expression was found if the transfected hESCs were cultured in self-renewal maintaining medium. By western blot, we observed the cleaved Notch1 protein (cN1) peaked on day 2 and diminished on day 3 after EB formation (Figure 1E). In addition, DLL1 protein expression was also elevated 48 hr after differentiation (Figure 1F). These data provide strong evidence that Notch signaling is activated in the initial stage of hESC differentiation.

Notch activation at the single-cell level 48 hr after EB formation was also examined by using the anti-cN1 antibody (Figures 1G and 1H). We observed that $cN1^+$ cells were largely lacking Oct4 expression (Figure 1G), whereas many coexpress a lineage-commitment marker Nestin (Figure 1H). The data indicate that Notch activation in hESCs is associated with exiting from the undifferentiated state and differentiation commencement.

We next examined directly the effects of Notch signaling blockade on the growth of undifferentiated hESCs (Figures 1I and 1J). As compared to the GFP control, GSI-18-treated or DNMAML-transduced H9 hESCs generated greater numbers of total cells after 5 days of culture and a higher frequency of colony-forming (self-renewing) undifferentiated hESCs, resulting in a total of 2.5-fold more undifferentiated colonies (Figure 1I). Similar results were obtained with the H1 hESC line expressing DNMAML, although the GFP-transduced or parental H1 hES controls also grew quite robust. We further characterized these $DNMAML^+$ cells by examining the expression of undifferentiated ES markers Oct4 and Tra-1-60 (Figure 1J). The percentage of $Oct4^+Tra\text{-}1\text{-}60^+$ cells with DNMAML transduction was comparable to the control hESCs. Moreover, both $DNMAML^+$ H1 and H9 hESCs can be cultured for 30–40 passages and remain phenotypically normal (data not shown). Therefore, the observed low-level Notch/CBF1 activity in undifferentiated hESC populations appears inessential for their self-renewal and is likely derived from a minor fraction of spontaneously differentiating or differentiated progeny in the standard hESC culture.

### Active Notch Signaling Initiates the Differentiation of hESCs

Conversely, we examined the consequence of induced Notch signaling activation in hESCs, either by exposure to the Notch ligand DLL1 or by enforced expression of *ICN1* or *HES1*

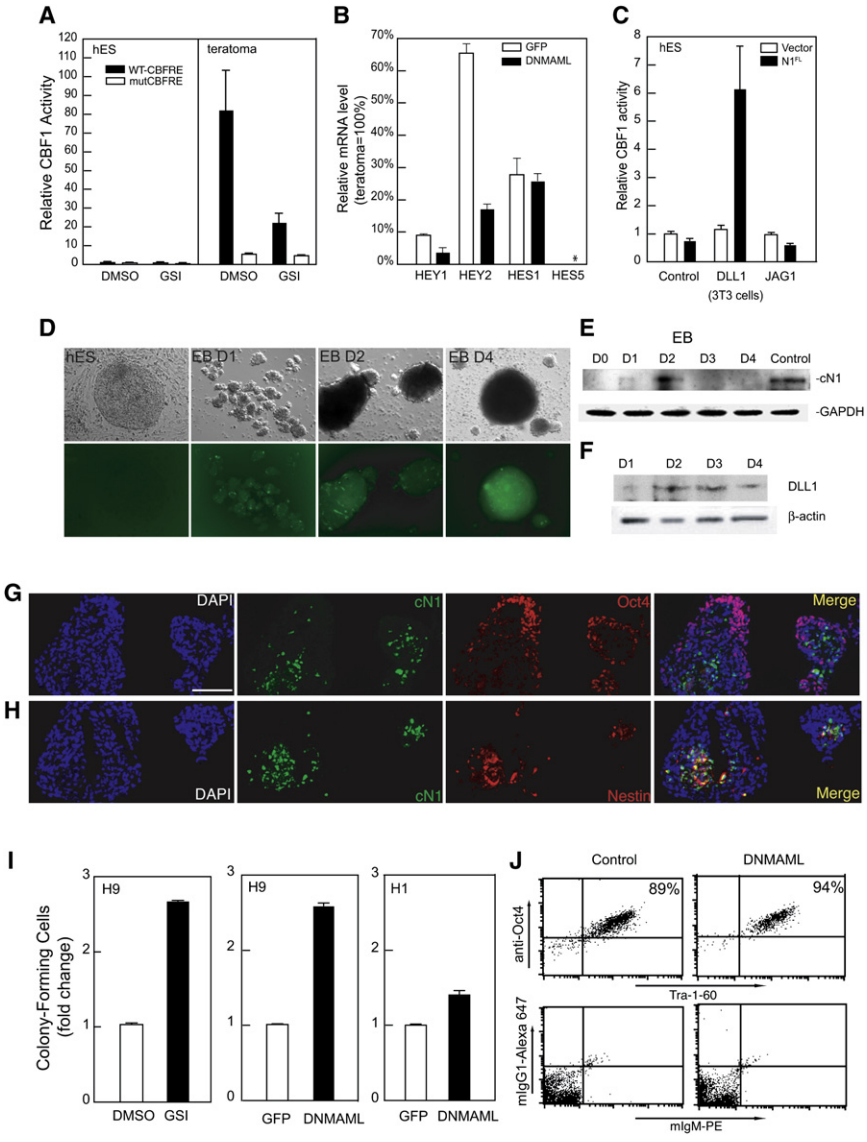


**Figure 1. Notch Activity in Undifferentiated and Differentiating hESCs**

(A) Undifferentiated ESCs (H1) were cocultured with MEFs in the presence or absence of GSI-18 (10 μM) for 3 days. Dissociated teratoma-derived cells were cultured in the same manner. The cells were then transfected with CBFRE-Luc construct together with β-gal expression plasmid overnight and subjected for Luc assays.

(B) Gene expression of representative Notch downstream target in H9 hESCs transduced with either DNMAML or the parental control lentivector. The relative level of each gene in the teratoma was defined as 100%. The asterisk indicates a very low level of *HES5* expression in GFP and DNMAML.

(C) H1 hESCs were overlaid on 3T3 cells expressing DLL1 or JAG1 for 1 day before transfection. CBFRE-Luc with full-length *Notch1* expression vector (pBOS-$N1^{FL}$) or parental vector was cotransfected into cells.

(D) CBFRE-GFP was electroporated into H1 cells. Except for an aliquot of cells continuously cultured under the ES culture condition, remaining transfected cells were induced to form EB in the presence of 20% FBS.

(E and F) EBs derived from H9 hESCs were collected daily for protein isolation. The expression of cleavage Notch1 (cN1) (E) and DLL1 protein (F) was detected by western blot. The proteins of mouse E14 germinal eminence were served as positive control for cleavage Notch1 (E).

(G and H) cN1(G and H), Oct4 (G), and Nestin (H) expression at the single cell level (H1 hESCs) 2 days after EB formation. Similar results were obtained in H9 EBCs. Scale bar, 100 μm.

(I) Self-renewal capacity in hESCs in which endogenous Notch activity was blocked by either DNMAML or GSI-18. hESCs were plated in 24-well Matrigel-coated plate at a density of $3 \times 10^4$ per well and cultured in the presence or absence of GSI-18 (20 μM) for one passage of 5 days. Cells were counted at the end of each passage. The frequency of colony-forming undifferentiated hESCs ($AP^+$) per 1000 harvested cells were also assayed after plating them on MEFs in 96-well plates for 5 to 6 days. Relative levels of colony-forming (CF) abilities were calculated by multiplying frequency of $AP^+$ colonies by the total cell number before colony assay. The level of CF ability of either $DNMAML^+$ or GSI-treated cells was expressed relative to the mean of the control ESCs, which was arbitrarily defined as 1. These experiments were repeated 3–6 times, and the pooled results are plotted ($n \geq 15$).

(J) The percentages of $Oct4^+Tra\text{-}1\text{-}60^+$ control or $DNMAML^+$ H1 cells were determined by FACS analysis. A representative data set from three independent experiments was shown.

transgene (Figure 2). First, hESCs were cocultured with 3T3-DLL1 or control 3T3 cells in undifferentiated hES culture medium (Figure 2A). hESCs cultured on 3T3-DLL1 cells for 4 days generated significantly fewer alkaline phosphatase positive ($AP^+$) undifferentiated colonies (Figure 2A; Figure S4). We further confirmed this observation by culturing hESCs with purified DLL1 (Varnum-Finney et al., 2000). The purified DLL1 as an IgG fusion protein ($DLL1^{ext\text{-}IgG}$) or IgG control was immobilized in Matrigel, which provides a feeder-free substrate. Eight days after treatment, comparable numbers of cells were harvested (Figure S5A). About 13% of DLL1-treated hESCs ($GFP^+$) lost Oct4 expression, whereas only 2% of cells were Oct4 negative in the control IgG group (Figure 2B). The number of undifferentiated hES colonies was reduced by >10-fold after the DLL1 treatment in a more stringent $AP^+$ colony-forming assay (Figure 2C; Figure S4B).

We also attempted to obtain stably transduced hESCs using lentiviral vectors coexpressing GFP and either ICN1 or HES1. Although transduced ($GFP^+$) ESCs were observed initially, ICN1-transduced cells (data not shown) and HES1-transduced cells disappeared after a few passages (Figure S6). In contrast, 100% of transduced cells in the control (GFP or ID1) groups remained $Oct4^+$ (Figure S6).

To better assess the effects of ICN1 or HES1 overexpression in hESCs, we utilized lentiviral vectors expressing a form of ICN1 as an estrogen receptor (ER) fusion protein (ICN1-ER; Figures S2A and S2B) and HES1-ER (Yu et al., 2006). In the absence of 4-hydroxytamoxifen (4HT) induction, ICN1-ER- or

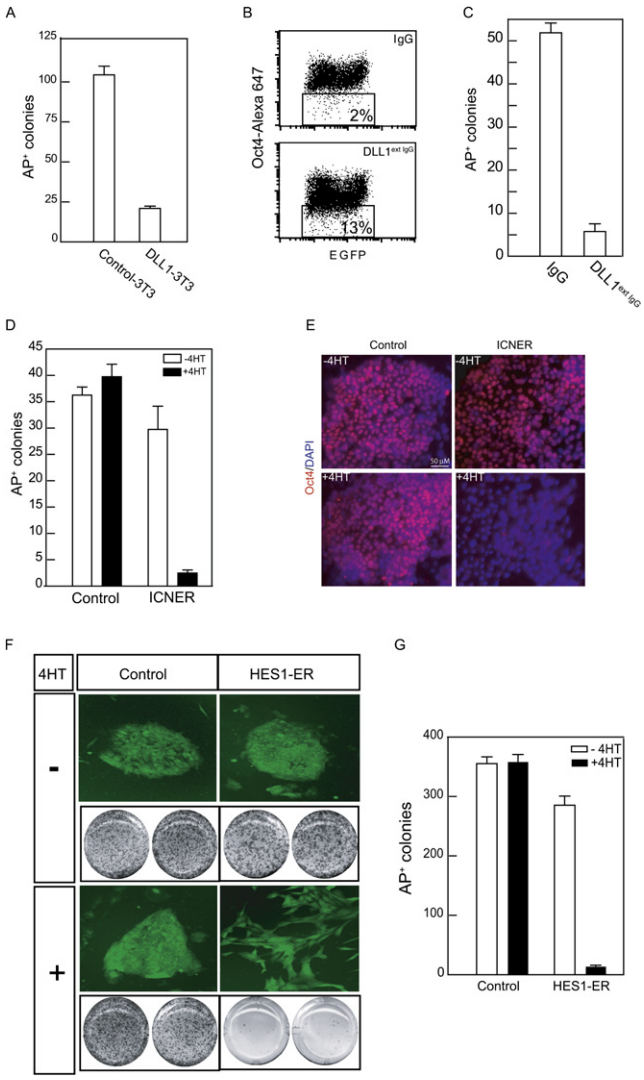


**Figure 2. Active Notch Signaling Initiates Lineage Commitment of hESCs**

(A) H9 ESCs were overlaid on 3T3 cells in the presence of MEF CM for 4 days. On Day 4, 1/15 of the trypsinized cells were replated on MEFs in 96-well plate for quantitative colony formation (n = 8 per group). A representative data set from three independent experiments is shown.

(B and C) $10^5$ of GFP$^+$ hESCs (H1) were plated in the 6-well plates precoated with 10 μg/ml of DLL1$^{ext\text{-}IgG}$ or control human IgG proteins. On Day 8, the percentage of Oct4$^+$ cells was measured by FACS (B). The harvested cells were further plated onto MEFs at a density of $2 \times 10^4$ per well for colony forming assay (C) (n = 8). Three independent experiments were performed.

(D and E) H9 cells stably transduced with parental or ICNER lentivector were cultured on Matrigel in the absence or presence of 4HT (200 nM) for 6 days. 2000 of trypsinized ESCs were replated for colony forming assay (D). In an independent experiment, 8 day treated cells were stained for Oct4 antibody (E).

(F and G) Control or HES1-ER$^+$ hESCs (H1) were cultured on Matrigel in the presence or absence of 4HT (200 nM) for two passages. Then, dissociated cells were plated in triplicates at a density of $10^4$ in 12-well of Matrigel-coated plates. After 4 days of culture, colonies were stained for the AP (F) and counted (G). Three independent experiments were performed.

HES1-ER-transduced hESCs had normal morphology and similar self-renewal ability comparable to the control GFP vector (Figures 2D–2G). Upon induction with 4HT, however, ICN1-ER$^+$ ESCs lost 92% of their colony-forming ability after one passage (Figure 2D). The Oct4 expression was also diminished in the majority of the ICN1-ER$^+$ cells (Figure 2E). Similarly, activation of HES1 by 4HT in hESs coexpressing HES1-ER and GFP transgenes led to a significant phenotypic change (Figure 2F). Eventually, the majority of cells lost their colony-forming ability after 10 days of 4HT induction (Figure 2G).

We monitored closely the numbers of viable and apoptotic cells after Notch activation. Similar numbers of cells were observed in both IgG and DLL1$^{ext\text{-}IgG}$ treated hESCs (Figure S5A). The total number of viable cells or the percent viable cells (Annexin$^-$/7AAD$^-$) in the ICN1-ER$^+$ hES group was similar to those of the control groups (Figures S5B and S5C). Thus, the reduction of undifferentiated hESCs after Notch activation primarily results from cell differentiation rather than decreased proliferation or increased apoptosis.

## A Pulse of Notch Signaling Is Required for hESCs to Form the Progeny of All Three Embryonic Germ Layers

Next, we examined the role of Notch signaling in the differentiation commitment of hESCs through EB formation. In the control hESC group (GFP$^+$), the size of aggregates increased with time, and more structured (cystic) EBs were visible after 3 days (Figure 3A). However, hESCs in which Notch signaling was blocked by DNMAML formed cystic EBs poorly, despite the fact that they appeared normal in their undifferentiated hESC state. To exclude the possibility that the observed effects might be due to increased apoptosis in Notch inhibited cells, we examined the cells for signs of apoptosis before and after the switch of culture conditions. No differences were observed in DNMAML$^+$ cells, as compared to controls (Figure 3B).

To quantify the spectrum and degree of differentiation, EBs were collected at different stages and analyzed by RT-PCR for the expression of representative markers of various embryonic cell lineages. After 5 days, the levels of *PAX6* (ectoderm marker), *AFP* (endoderm marker), and *CD34* and *SCL/TAL-1* (mesoderm markers) were sharply elevated in the control hESCs from undetectable at day 0 (data not shown) to levels comparable (or higher for AFP) to those of teratoma cells (Figure 3C). However, the expression of these markers was significantly lower in the DNMAML-transduced group, suggesting that differentiation into the progeny of all three embryonic germ layers is blocked. We also analyzed the expression of *NANOG* and *OCT4* (undifferentiated ESC markers) in these cells. The DNMAML$^+$ cells expressed more than 20-fold higher levels of *NANOG* or *OCT4*, as compared to that of the control group, further indicating that blocked Notch activity inhibited ESC differentiation. Similar results were found in both H9 and H1 cells treated with GSIs (Figures 3F and 4A).

Inefficient EB formation observed with DNMAML$^+$ hESCs may result from poor cell-cell contact, which in turn might affect differentiation within EBs. Thus, we used a method to generate EBs that forces cell-cell contact in 96-well microtiter plates (Ng et al., 2005). In marked contrast to the control group, few of DNMAML$^+$ EBs became cystic after 12 days of culture (Figure 3D). This reduced cystic EB formation was also seen in the wild-type hESCs after GSI (GSI-18 or GSI-2) treatment. In the presence of DMSO vehicle alone, 70% of EBs transduced with either control or HES1-ER lentivector was cystic (Figure 3E).

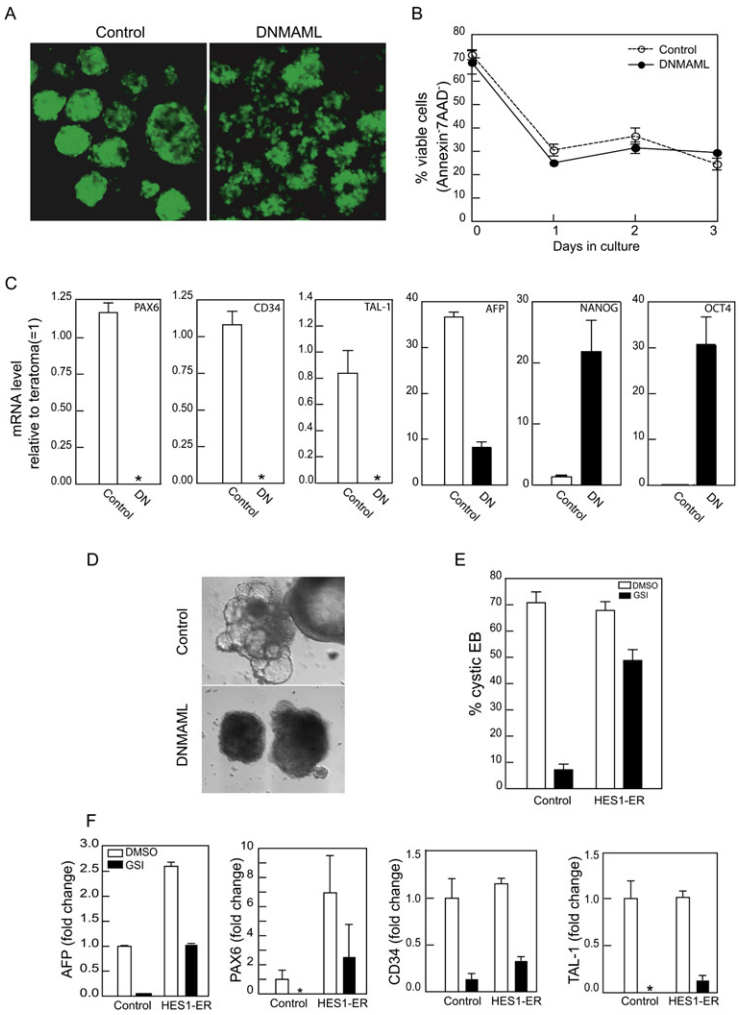


**Figure 3. Notch Signaling Is Required for the Differentiation of hESCs into the Progeny of the Three Germ Layers In Vitro**

(A–C) hESCs (H9) were cultured in the presence of 20% FBS to induce EB formation. (A) EBs of day 3 were shown. (B) The percent of viable cells was determined. Two independent experiments were performed with duplicates per group in each experiment. (C) Gene expression in day 5 H9 EBCs. The expression level of each gene in teratoma was arbitrarily set as 1.

(D–F) 1000 H1 cells were forced to aggregate after centrifugation in 96-well plate. 1 to 2 EBs were formed in each well after 12 day culture (D). (E) ESCs were differentiated in the presence or absence of 10 μM of GSI-2. 200 nM of 4HT were added into all cultures to induce the functional HES1 expression. Cystic and total day 12 EBs were documented. Three independent experiments were grouped to present here. (F) Enforced expression of HES1 partially restored the normal EB (day 12) differentiation blocked by GSI. The expression of each gene in DMSO treated control EB was normalized to 1. (*$Ct$ = 0 after 40 cycles).

However, continuous treatment with a GSI inhibited EB maturation (<10% of total EBs were cystic) in the control group. The inhibition of cystic EB formation by GSI was largely rescued by the *HES1* gene expression: ~50% of the HES1-ER$^+$ EBs remained cystic at the end of a 12 day culture in the presence of 4HT induction.

Next, we analyzed the expression of lineage markers of the three embryonic germ layers in the day 12 EBs formed in the presence or absence of GSI, from either control or the HES1-ER-transduced hESCs (Figure 3F). As expected, GSI-treated cells transduced with the control lentivector showed severely reduced expression of all the lineage markers used (*AFP*, *PAX6*, *CD34*, and *TAL-1*). In contrast, HES1-ER activation by 4HT resulted in the partial restoration of expression of the differentiation markers in GSI-treated cells, suggesting that the observed GSI effect in this system is Notch signaling specific. Collectively, our data demonstrate that Notch signaling is required for lineage commitment to form cell derivatives of the three embryonic germ layers.

A pulse of Notch signaling observed within 48 hr of EB differentiation (Figures 1D–1F) may be essential for lineage commitment of hESCs. To test this hypothesis, GSI-18 (or DMSO vehicle) was added into the culture at either the beginning of EB formation or 2 days later. In contrast to the significant changes observed if GSI treatment started at day 0 (Figure 4A), starting GSI treatment at day 2 had little effects on EB maturation or gene expression patterns in EB-derived cells at day 6 (Figure 4B). The data confirmed that a pulse of Notch signaling within first 48 hr of EB formation is required for hESC to differentiate and commit to the three embryonic lineages.

## Notch Signaling Blockade Preserves Undifferentiated hESCs after Induced Differentiation In Vitro

High levels of *NANOG* and *OCT4* mRNA expression were detected after differentiation induction in hESCs with reduced Notch activity (Figures 3C and 4A), indicating that some ESCs remained undifferentiated within EBs. We stained day 6 EBs to detect the level of nuclear Oct4 protein (Figure 4C). Significant numbers of Oct4$^+$ cells remained in the DNMAML group. In contrast, only a few scattered Oct4$^+$ cells were observed in control ESC-derived EBs.

Next, we assessed the content of remaining undifferentiated hESCs in the DNMAML group during a prolonged differentiation culture. Day 12 EBs were dissociated into single cells and plated on feeder cells in culture conditions designed to maintain undifferentiated hESCs. The number of AP$^+$ colonies derived from the DNMAML$^+$ group was 4-fold higher than that of the control EBCs (Figure 4D), indicating that reduced Notch activity prevented the undifferentiated hESCs from entering differentiation. To induce further in vitro differentiation, day 12 EBs were transferred onto mouse OP9 cells and cultured for an additional 7 days. Then, all cells were harvested for immunostaining for Tra-1-60 (an undifferentiated marker) and Tra-1-85, a marker unique for human cells (Figure 4E). After a two-step, 19 day differentiation, only 11%–13% of untransduced or parental GFP-transduced cells remained Tra-1-60$^+$. In contrast, 54% of DNMAML$^+$ cells still expressed Tra-1-60.

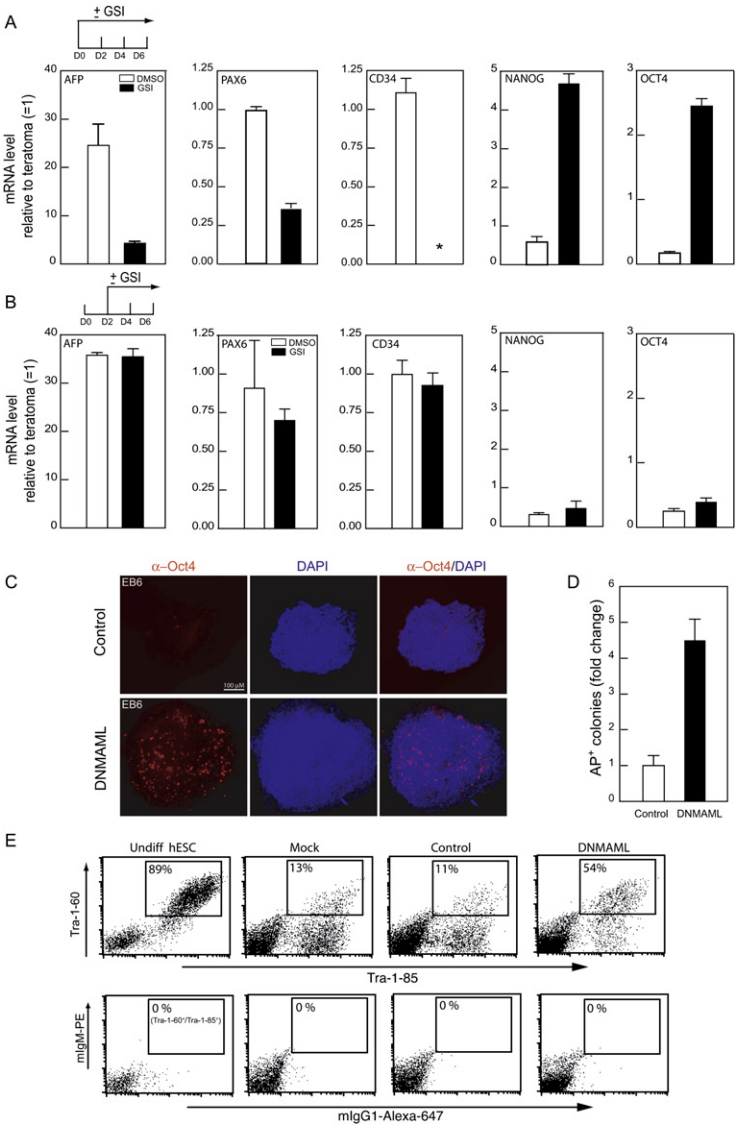


**Figure 4. A Pulse of Notch Signaling Is Essential for Lineage Commitment of hESCs**

(A and B) H9 ESCs were induced to differentiate into EBs in the presence of 20% FBS for 6 days. Continuous treatment of GSI-18 (20 μM) or DMSO was started on Day 0 (A) or Day 2 (B). The mRNA level of the day 6 EBCs was measured by qRT-PCR and expressed relative to the mean of the teratoma (which was normalized to 1). * indicates *Ct* = 0 (undetected) after 40 cycles.

(C–E) Blocking Notch signaling preserves uncommitted hESCs during differentiation in vitro. (C) Day 6 whole EBs (H9) were stained for Oct4 antibody. (D) 1000 of dissociated day 12 EBCs were cultured on MEFs in 96-well plate for 7 days. Colonies were further stained for AP. The number of colonies derived from control EBCs were normalized to 1. (E) Day 12 EBs (H1) were cultured on plain OP9 cells for 6 days. Cell mixtures were then isolated and costained with anti-Tra-1-60 (ESC marker) and Tra-1-85 (human cell marker) antibodies.

## Active Notch Signaling Enhances Hematopoietic Differentiation after Mesoderm Commitment

Next, we examined whether Notch activation by HES1 overexpression enhanced hematopoietic differentiation after mesoderm commitment (Figure 5). HES1-ER$^+$ EBs aggregated in the presence of 4HT for 12 days generated more hematopoietic colonies than control EBs (Figure 5A). When EB-derived cells were allowed to further differentiate on OP9 stromal cells for 7 days, 39% of the cells in the HES1-ER group were CD34$^+$CD45$^+$. In contrast, only 13% of the cells were CD34$^+$CD45$^+$ in the control (GFP only) group (Figures 5B and 5C). Notably, 4HT was added only during the first stage of differentiation (EB formation) to activate the HES1-ER transgene, but not in the two subsequent hematopoietic assays. If exposure to 4HT was continued during the hematopoietic assays, however, cell proliferation was reduced (data not shown). This is consistent with our previous observation that continued HES1 overexpression inhibited postnatal mouse and human hematopoietic progenitor cell proliferation (Yu et al., 2006). Our results demonstrated that controlled activation of Notch signaling can be used to direct differentiation of hESCs to a specific lineage under appropriate culture conditions.

## Blocking Notch Signaling Favors Trophoblastic Lineage Commitment upon Differentiation Induction

In addition to cells derived from the three embryonic germ layers, hESCs can differentiate into trophoblasts by either EB formation (Gerami-Naini et al., 2004) or BMP4 induction (Xu et al., 2002). To examine the potential role of Notch signaling in trophoblastic differentiation, we first analyzed the expression of trophectoderm markers (i.e., *CDX2*, chorionic gonadotropin A and B [*CGA*, *CGB*]) within EBs (Figures 6A–6C). Levels of mRNA of these genes were significantly elevated in differentiating DNMAML$^+$ EBCs (H1) compared to the control groups (Figures 6A–6C). Similar results were observed with H9 hESCs, when whole EBs were stained for trophoblastic markers such as TROMA-I (Figure 6D). More TROMA-I$^+$ cells were observed in the peripheral zones of DNMAML$^+$ EBs than that of control EBs (Figure 6D). We also measured by FACS what proportion of the cells in EBs (day 12) expressed trophoblastic markers. TROMA-I or β-hCG was expressed in 5% to 6% of DNMAML$^+$ EBCs. In contrast, <1% of the control EBCs expressed trophoblastic markers (Figure 6E).

The effect of Notch blockade on BMP4-induced trophoblastic differentiation was also examined in several transduced hESC lines (Figures 6F–6H). Cells were plated at the same density and induced by BMP4 for 10 days. Supernatants were then collected for measuring β-hCG hormone production by ELISA. Relative to cells transduced with the control lentivector, the HES1$^+$ cells produced only 20% of the hCG protein (Figure 6G). In contrast, hESCs transduced with a HES1 DNA-binding mutant (ΔBHES1) showed normal hCG production. Reduction in hCG production was also observed in ICN1$^+$ ESCs (Figure 6F). Blocking Notch activity using a GSI resulted in a 2-fold increase in hCG production in control cells, but not in ICN1$^+$ cells. We also

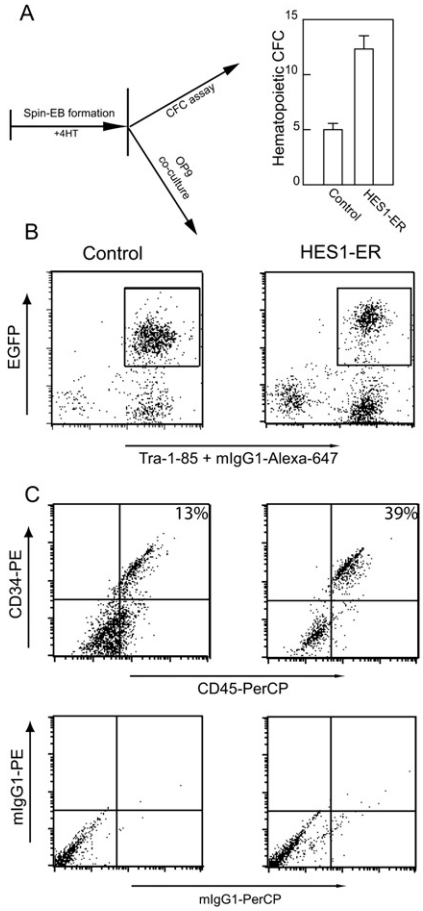


**Figure 5. Transient Activation of Notch/HES1 Signaling Promotes Hematopoietic Differentiation**

(A) 3000 of control or HES1-ER$^+$ ESCs (H1) were forced to aggregate in 96-well plates in the presence of BMP4 (10 ng/ml), VEGF (5 ng/ml), Flt-3 ligand (5 ng/ml), SCF (20 ng/ml), IL-6 (5 ng/ml), and 200 nM of 4HT. 1.7 × $10^4$ dissociated EB12 cells were plated in methylcellulose supplemented with IL-3, GM-CSF, and Epo for hematopoietic colony assay.

(B and C) Whole EBs (day 12) were transferred onto OP9 cells in the absence of 4HT. Seven days later, cells were harvested for FACS analysis (C). Human cells were gated based on the positive GFP and Tra-1-85 staining shown in (B).

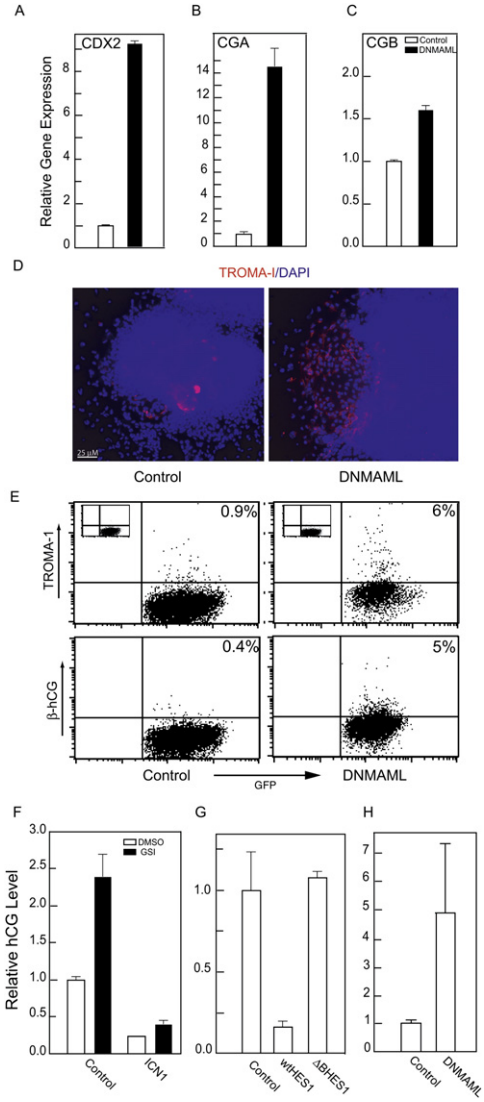


**Figure 6. Blockade of Notch Signaling Favors Trophoblastic Differentiation of hESCs**

(A–C) Gene expression of trophectoderm markers in day 7 EBs (H1). Control EBs (n = 3); DNMAML$^+$ EBs (n = 4).

(D) TROMA-I staining in day 14 EBs (H9).

(E) Day 12 EBs (H9) were dissociated into single cells and stained with either TROMA-1 or β-hCG antibody for FACS analysis.

(F–H) $10^5$ of hESCs were plated in the presence of BMP4 (50 ng/ml) for 10 days. The supernatants were collected for hCG measurement. The cell lines examined were cells transduced with parental, ICN1-ER (F), HES1-ER, ΔBHES1-ER (G), and DNMAML lentivector (H). (F) 200 nM of 4HT was added during the 10 day culture to induce functional ER fusion protein expression in H1 cells. Cells were also treated with either DMSO (empty bar) or 10 μM of GSI-2 (filled bar). Three independent experiments were performed and data were grouped here.

analyzed hCG levels in the DNMAML$^+$ cells. The DNMAML$^+$ cells produced ~5-fold higher levels of hCG, as compared to the level measured in control groups (Figure 6H). Together, these results demonstrate that trophoblastic differentiation is inhibited by the activated Notch signaling.

## Blocking Notch Signaling Affects hESC Fate during In Vivo Differentiation

We next examined cell-fate choices of hESCs in vivo during teratoma formation from control and DNMAML-transduced hESCs. GFP (control) and DNMAML-transduced ESCs (both H1 and H9) were injected into mice. After 3 to 4 months, palpable tumors were excised. Both control and DNMAML groups formed tumors at similar rates, and no significant difference in size or weight was observed (data not shown). We examined whether DNMAML remained functional in teratoma cells by examining CBFRE-Luc activity after overexpression of ICN1. Interestingly, four out of seven teratomas in the DNMAML-transduced group showed a low level of CBF1 activity even after ICN1 activation, indicating an active DNMAML inhibition (DN-active) as expected. However, the remaining three teratomas in the DNMAML-transduced group showed a high CBF1 activity, comparable to control

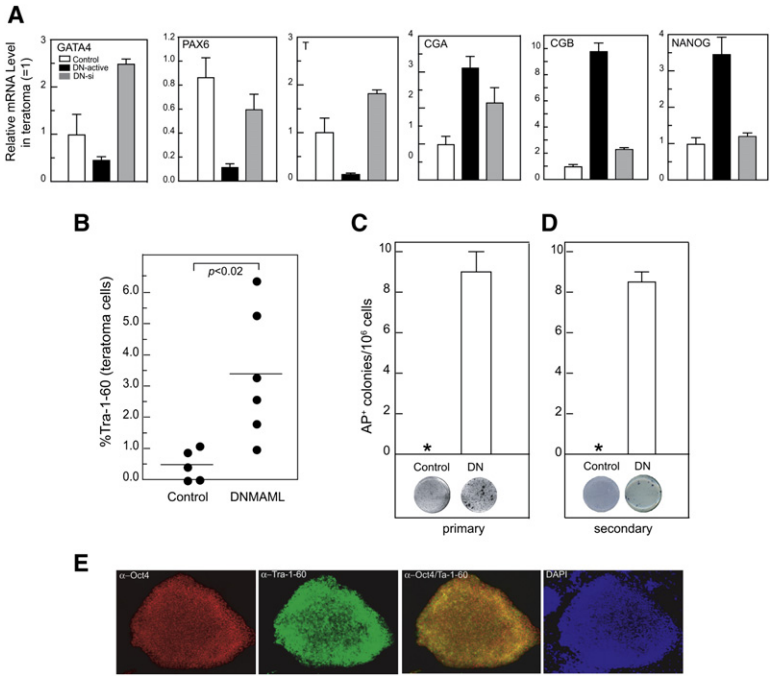


**Figure 7. Blocking Notch Signaling Alters hESC Differentiation In Vivo**

Control or DNMAML-transduced hESCs were injected intramuscularly into the *SCID/Beige* mice. Palpable teratoma was dissected into pieces for various assays. The induction of CBFRE-Luc activity by ICN1 was examined in dissociated DNMAML$^+$ tumor cells. The DNMAML tumor cells that showed restored ICN1-induced CBF1 activity was designated as silenced DNMAML cells (DN-si).

(A) qRT-PCR analysis of gene expression in teratoma cells (H9). The gene expression level of control teratoma cells was normalized as 1. (Control = 3; DN$^+$ = 4; DN-si = 3).

(B) Dissociated teratoma cells (H1) were immunostained for Tra-1-60 and Tra-1-85 antibodies and analyzed by FACS. Human cells were gated based on positive Tra-1-85 staining. (Control = 5; DNMAML = 6).

(C) $10^6$ of teratoma cells (H1) were replated onto MEFs in 6-well plate and cultured in ES media for 7–9 days. The survived cells were then stained for AP. (Control = 8, DNMAML = 9).

(D) In secondary colony assay, teratoma-derived primary colonies were collected and replated (ratio of 1:1) onto new MEF cells. The cells were cultured for 7 more days and stained with AP. Three control and three DNMAML teratoma samples were used in this assay. (Asterisks indicate no colonies formed).

(E) Characterization of a tertiary colony derived from DNMAML-transduced teratoma cells.

(GFP) teratomas (as shown in Figure 1A). This DNMAML-transduced subgroup is distinct from the four DN-active teratomas. It is likely that the DNMAML-mediated inhibition was silenced after several months in vivo in these three teratomas (designated as DN-si). We observed the expression levels of representative lineage/differentiation marker genes, *GATA4* (endoderm marker), *PAX6* (ectoderm marker), and *T* (Brachyury, mesoderm marker), were reduced in harvested DN-active cells. In contrast, the expression of these representative lineage markers in the DN-si teratoma cells was more similar to the control (GFP) teratoma cells (Figure 7A). In addition, the expression of trophoblastic markers was increased by >3-fold in DN-active cells compared to the control and DN-si groups. *NANOG* gene expression was 3.5-fold higher in the DN-active cells than those in the control or DN-si group, indicating that active DNMAML inhibition reduced the differentiation of hESCs in vivo.

Freshly isolated teratoma cells were also analyzed by FACS for the presence of cells that retained undifferentiated hESC phenotype (Tra-1-60$^+$) (Figure 7B). On average, 3.5% of the teratoma cells in the DNMAML group (n = 6) expressed Tra-1-60, whereas only about 0.5% of teratoma cells in the control group (n = 5) remained Tra-1-60$^+$. To directly evaluate the content of remaining undifferentiated hESCs within teratomas, $10^6$ of dissociated teratoma cells from the DN-active group were plated onto feeder cells under hESC culture condition. Seven to nine days later, undifferentiated hES colonies appeared, which were stained positive for AP activity (Figure 7C). No AP$^+$ colonies were generated from the control tumors. Furthermore, secondary AP$^+$ colonies were formed after the primary culture was replated on MEFs (Figure 7D). Tertiary hES-like colonies were also formed and Oct4$^+$ and Tra-1-60$^+$ (Figure 7E). In summary, an enhanced level of undifferentiated hESCs was observed in the teratoma in which DNMAML-mediated blockade remained active months after differentiation induction in vivo.

## DISCUSSION

Using both genetic and pharmacological approaches, we examined the role of Notch signaling in both self-renewal and differentiation of hESCs by a series of gain-of-function and loss-of-function studies. First, we confirmed that Notch signaling is inactive in undifferentiated hESCs, which is in agreement with a recent report (Noggle et al., 2006). Neither Notch cleavage nor CBF1 activity was detected (Figures 1C–1F). In contrast, Notch signaling was activated in differentiating hESCs. We observed that a transient wave of Notch signaling activation appeared after induced EB formation and peaked around 48 hr after differentiation (Figures 1D–1F). Notch activation in hESCs correlates the Oct4 expression reduction (Figures 1G, 2B, and 2E), the exit from the undifferentiated state and differentiation initiation (Figures 1G and 1H). We demonstrated that this wave of Notch signaling activation is essential for hESC lineage commitment to the progeny of the three embryonic germ layers (Figures 3A and 4A). However, the cells were able to differentiate to a level comparable with control cells if Notch signaling blockade was delayed for 48 hr (Figure 4B). Whether such a wave occurs during human embryonic development in vivo is of great interest to investigate.

Our data demonstrated that the Notch signaling pathway is inactive in undifferentiated hESCs, although a minor fraction of spontaneously differentiating or differentiated progeny cells displayed Notch/CBF1 activity. Short-term DLL1 stimulation (2 days) was not sufficient to trigger the endogenous Notch cleavage monitored by the CBF1 activity in undifferentiated hESCs, unless the additional exogenous $N1^{FL}$ cDNA was provided as well (Figure 1C). This implies that the levels of Notch receptors and ligands in undifferentiated hESCs are below the threshold of activation. It remains to be determined how Notch signaling is activated upon differentiation induction in hESCs, which are capable of responding to the exposure of an

exogenous Notch ligand for >2 days (Figure 2). There are several possibilities we can postulate. First, the pulse of Notch activation (Figure 1E) may result from increased cell-cell contact during EB or teratoma formation with increased Notch ligand density or ligand-receptor interactions. Second, the differentiation induction may increase expression levels or activities of Notch receptors and/or ligands. In fact, our data showed that DLL1 expression was significantly increased within the first 48 hr of EB differentiation relative to undifferentiated hESCs (Figure 1F). We also observed the increased *Notch1* receptor gene expression by about 1.5-fold in differentiated hESCs by qRT-PCR (Figure S1). Despite of the uncertainty of which and how many mechanisms are involved, our current data nonetheless suggest that Notch signaling plays an active role in hESC differentiation. Better understanding the regulation of Notch regulators in a cell context-specific manner may help us to better understand initial key events during the hESC lineage commitment.

In addition to the understanding of a critical role of Notch signaling pathway in the initiation of hESC lineage commitment, our study also suggests that manipulation of Notch signaling at a later stage of differentiation is likely to be useful in specification of hESC into tissue-specific progenitors. The promotion to the ectodermal-neural lineage by Notch activation was shown in mESCs and one hESC line (Lowell et al., 2006). The authors also showed that Notch activation in mESCs after the stage equivalent to the epiblast formation simultaneously suppressed nonectodermal commitment while promoting the ectodermal-neural lineage (Lowell et al., 2006). Here, we observed that Notch signaling promotes differentiation of hESCs to generate the progeny of not only the ectoderm lineage, but also mesoderm and endoderm lineages. For example, we found that transiently overexpressed HES1 enhanced production of hematopoietic cells derived from mesoderm precursors (Figure 5).

Our current studies benefited from our improved ability to transduce hESCs efficiently and stably with lentiviral vectors. However, we noticed that, in every case, transgene expression driven by a housekeeping gene promoter was gradually silenced in hESCs in the absence of continuous selection (Zhou et al., 2007). The silencing of DNMAML-GFP transgene expression was also observed in DNMAML-GFP-transduced hESCs after initial selection of $GFP^+$ hESCs. Three teratomas derived from the DNMAML-transduced cells found that the inhibitory effect of DNMAML on Notch signaling was lost probably due to gene silencing. Interestingly, the expression of all the three embryonic germ layer markers in these three teratomas was comparable with the control cells (Figure 7). In contrast, the markers were expressed at a reduced level in DNMAML-active teratomas. This indicates that hESCs in which endogenous Notch signaling was initially blocked were able to retain differentiation ability once Notch signaling activation was restored.

Our data support an emerging notion that a GSI with low cytotoxicity can be used to enhance the maintenance of undifferentiated hESCs by preventing spontaneous differentiation occurring commonly in current culture systems (Figures 1I and 1J). It was postulated previously that Notch signaling is associated with or important to self-renewal of hESCs (Androutsellis-Theotokis et al., 2006; Fox et al., 2008). The latter postulation was based largely on mRNA expression data and the use of GSI such as DAPT (Androutsellis-Theotokis et al., 2006) and L685458 (Fox et al., 2008) that reduced numbers of total and colony-forming hESCs. The reduction of hESC growth by DAPT was also observed by us (Figure S3) and others (Noggle et al., 2006), possibly resulting from nonspecific toxicity of the chemical. In fact, analysis of survived hESCs after DAPT treatment revealed that they are more homogenous as undifferentiated hESCs (Noggle et al., 2006). A newer GSI we used, GSI-18, showed no toxicity in contrast to DAPT (Figure S3) and actually enhanced the growth of undifferentiated hESCs by preventing spontaneous differentiation (Figure 1I). Corroboratively, there is no convincing evidence that the Notch pathway is involved in the self-renewal of mESCs, either. It is well known that embryos deficient in the genes encoding various Notch pathway components can develop to midgestation stages but die around embryonic day 8–11 (Yoon and Gaiano, 2005). Furthermore, Notch1- or CBF1-deficient mESCs can be established and maintained with undifferentiated phenotypes, although they show abnormalities after differentiation induction (Nemir et al., 2006).

We also examined the Notch effect on trophoblastic differentiation of hESCs. Surprisingly we found that blockade of Notch signaling promoted the expression of trophoblastic markers (Figures 6A and 7B) and increased the proportion of cells expressing trophoblastic markers during in vitro differentiation (Figures 6D and 6E). Overexpression of *ICN1* or *HES1* resulted in inhibition of hCG production (Figures 6F–6H). Taken together, our data demonstrate that Notch activation in hESCs promotes their commitment to form the progeny of all three embryonic germ layers but inhibits trophoblast differentiation, an alternative cell-fate choice at this stage. The observed fate choices between two major differentiated lineages of hESCs resembles the well-known phenomenon of ''lateral inhibition,'' in which Notch activation helps to generate more than one type of committed progeny within an initially uniform stem/progenitor cell population (Bray, 2006). We propose a simple model to illustrate the essence of our findings on the role of Notch signaling on hESC-fate determination (Figure S7). This model is consistent with a common theme of Notch signaling in stem cell regulation, emerged from various stem cell systems (Ohlstein and Spradling, 2007). Although cell context dependent, Notch signaling appears primarily involved in the specification of cell fate instead of acting directly on stem cell survival, proliferation, or maintenance. A detailed analysis of the Notch pathway and its interactions with other regulators will help us to better understand the mechanism of cell-fate determination of hESCs.

We observed differences between hESCs and mESCs in response to Notch activation, as to other cues such as BMP4. Mouse ESCs can tolerate overexpression of *ICN1* (6-fold) without altering their undifferentiated phenotype under culture conditions designed to promote self-renewal and maintain pluripotency (Lowell et al., 2006). In contrast, hESCs lost their self-renewal capacity and differentiated by either sufficient exposure to exogenous DLL1 ligands or overexpression (~5-fold) of *ICN1* or *HES1* (Figure 2). Other differences between hESCs and mESCs were revealed by induced differentiation. In the absence of two key self-renewal factors for mESCs, LIF and BMP4, active Notch signaling favors mESC differentiation into a neuroectodermal fate (Lowell et al., 2006). Interestingly, shutting down of Notch signaling is required for cardiogenesis from mES-derived EBs (Nemir et al., 2006; Schroeder et al., 2006). For hESCs, we

show that a pulse of Notch signaling is initiated upon differentiation induction and is essential for hESC commitment to form the progeny of all three embryonic germ layers. We also found that blockade of the Notch pathway increased the proportion of trophoblast cells as well as undifferentiated hESCs. Currently, we do not know whether the promotion of trophoblastic differentiation by Notch pathway blockade is a direct or indirect effect.

In summary, this is the first comprehensive study to define the role of Notch signaling in the decision of hESCs to self-renew or differentiate and in cell-fate choices between embryonic and trophoblastic cell lineages upon differentiation induction. For the initial cell-fate determination of undifferentiated hESCs, active Notch signaling was only required for the commitment to form cells of all the three embryonic germ layers, but not for self-renewal or trophoblastic differentiation. Our data shed light on one mechanism governing hESC-fate determination and open the possibility of manipulating Notch signaling to more efficiently promote directed differentiation of hESCs or other forms of pluripotent stem cells into therapeutically relevant cell types.

## EXPERIMENTAL PROCEDURES

### Human ESC Culture

H1 and H9 hESC lines were obtained from WiCell Research Institute. See the Supplemental Data for detailed culture conditions.

### Teratoma Formation

hESCs were scraped from the plate and resuspended in 250 μl of HBSS and injected into the leg muscle of 4-week-old *SCID*/Beige mouse. Palpable teratoma (after 3 to 4 months) was excised for further analysis.

### Transduction of hESCs

Concentrated lentiviruses were added into the 80% confluent cells in the presence of polybrene (4 ng/ml, H9268, Sigma-Aldrich). After 6–8 hr, cells were replated on MEF cells or supportive human feeder cells expressing drug selection genes. Cells were either sorted based on GFP fluorescence or selected by the hygromycin (10 μg/ml), puromycin (1 μg/ml), for 2 to 3 passages.

### Transfection of hESCs

Lipofection and electroporation in hESCs were performed as described previously (Cai et al., 2007).

### γ-Secretase Inhibitors and CBFRE Reporter Constructs

GSI-18 (synthesized by Dr. Yue-Ming Li), GSI-2 (cat. no. 565755, EMD Biosciences) and DAPT (cat. no. D5942, Sigma-Aldrich) were used in the study. CBFRE containing eight copies of wild-type or mutant CBF1 binding sites (Hsieh et al., 1996) were cloned into pTA-Luc construct (Clontech Laboratories).

### Statistical Analysis

Data plotted are typically expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using GraphPad Prism version 4.0 (GraphPad Software). Significance of differences was examined using the Student's t test (two-sided, unequal variance).

## SUPPLEMENTAL DATA

The Supplemental Data include one table, seven figures, and Supplemental Experimental Procedures and can be found with this article online at http://www.cellstemcell.com/cgi/content/full/2/5/461/DC1/.

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